HHS Public Access

Author manuscript

Curr Opin Neurobiol. Author manuscript; available in PMC 2018 June 01.

Published in final edited form as:

Curr Opin Neurobiol. 2017 June ; 44: 80–88. doi:10.1016/j.conb.2017.03.009.

Attacking sleep from a new angle: contributions from zebrafish

Grigorios Oikonomou and **David A. Prober**

Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125, USA

Abstract

Sleep consumes a third of our lifespan, but we are far from understanding how it is initiated, maintained and terminated, or what purposes it serves. To address these questions, alternative model systems have recently been recruited. The diurnal zebrafish holds the promise of bridging the gap between simple invertebrate systems, which show little neuroanatomical conservation with mammals, and well-established, but complex and nocturnal, murine systems. Zebrafish larvae can be monitored in a high-throughput fashion, pharmacologically tested by adding compounds into the water, genetically screened using transient transgenesis, and optogenetically manipulated in a non-invasive manner. Here we discuss work that has established the zebrafish as a powerful system for the study of sleep, as well as novel insights gained by exploiting its particular advantages.

Why zebrafish?

By the start of this century, the zebrafish was a well-established model for developmental studies. As has often been the case for model systems, the use of zebrafish has recently expanded from studies of development to behavior. Zebrafish are vertebrates whose nervous system and neuropharmacology is highly conserved with that of mammals. Crucially for the study of sleep, zebrafish are diurnal, similar to humans, and unlike commonly used murine systems. The generation time of zebrafish is similar to that of mice, and more genetic tools are currently available for mouse studies, but the power of zebrafish comes from the ability to work with larvae, as opposed to adults. A single zebrafish mating routinely provides 200–300 embryos, which by the 5th day of development have developed into independent larvae that exhibit robust wake/sleep cycles (Box 1). The small size (~4 mm) of these larvae enables their behavior to be tracked in 96-well plates (Figure 1A), allowing for robust statistical analysis. Furthermore, the advantages that make zebrafish an excellent developmental model system (external development, optical transparency, facile transgenesis and drug administration) are also applicable and useful in the study of the development and function of sleep circuits. Thus, zebrafish complement more established model systems for the study of sleep by straddling the gap between simple and efficient invertebrate model systems, and more expensive and demanding mammalian models. Here we focus on recent

Corresponding author: Prober, David A (dprober@caltech.edu).

contributions of zebrafish to our understanding of sleep regulation, with a focus on work that highlights the particular advantages of this system.

Box 1

Sleep in zebrafish

The gold standard for determining and quantifying sleep states in mammals has been the combination of electroencephalography (EEG) and electromyography (EMG); specific EEG profiles correlate with different sleep states, while REM sleep is characterized by lack of muscle tone. These approaches are not practical for the small zebrafish larvae, or for invertebrate model systems. Instead, researchers rely on 3 behavioral criteria to differentiate sleep from inactivity. **(1) Behavioral quiescence.** This state must be rapidly reversible, thus distinguishing it from paralysis or coma, and is usually regulated by the circadian clock. **(2) Increased arousal threshold.** In contrast with awake but inactive animals, sleeping animals require stimuli of higher intensity to respond at the same frequency as awake animals. **(3) Homeostatic control.** Sleep pressure increases with time spent awake, and following sleep deprivation there is an increase in sleep duration to compensate for lost sleep. Zebrafish larvae satisfy all three criteria: most sleep occurs during the dark phase of the circadian cycle [70], and larvae exhibit increased arousal threshold after 1 or more minutes of inactivity [21••], as well as sleep rebound after sleep deprivation caused by either mechano-acoustic stimulation [2••] or by constant water flow [71].

Although these behavioral criteria have been used to demonstrate that zebrafish larvae sleep, the lack of a real-time indicator of sleep, such as the EEG/EMG combination used in mammalian systems, presents significant challenges. Until a real-time reporter of sleep states is developed for zebrafish larvae, the community must be careful in interpreting locomotion-based results. Such precautions include common good scientific practices such as using a variety of approaches (genetics, pharmacology, direct manipulation of neuronal activity), using arousal threshold assays, investigating the interactions of potential novel sleep systems with previously established sleep centers, as well as comparing zebrafish results with studies in other vertebrate and invertebrate systems.

Melatonin

Similar to mammals, melatonin in zebrafish is produced by the pineal gland at night, under the control of the circadian clock [1]. Administration of exogenous melatonin was shown to induce sleep in larval zebrafish [2••] similar to humans [3], in the first demonstration of conservation of sleep regulation between zebrafish and humans at the molecular level. In the same seminal study [2••], it was also demonstrated that rest in zebrafish larvae fulfills the behavioral criteria of sleep (Box 14), thus establishing zebrafish as a sleep model. Although melatonin is produced at night (rest period) in diurnal animals such as humans and zebrafish, it is also produced at night (active period) in nocturnal rodents [4]. Melatonin has a sleep-promoting effect in several diurnal species, including humans [3,5], but does not induce sleep in nocturnal animals [3]. These observations suggest that nocturnal animals are not

ideal models to understand how melatonin regulates sleep in diurnal vertebrates such as humans. Further complicating matters, most commonly used laboratory mouse strains do not produce melatonin [4] (melatonin promotes seasonal reproduction patterns, so as wild mice strains were adapted to the lab there was presumably an unintended selection for melatonin deficient mutants). This makes zebrafish a particularly attractive system for investigating the role of melatonin in sleep.

Historically, the main avenue for depletion of endogenous melatonin had been pinealectomy, a crude technique likely to produce artifacts due to its invasiveness, variability among labs and different species, and uncharacterized, melatonin-independent roles of the pineal gland in animal physiology. Recently however, considerable progress in clarifying the function of melatonin in sleep was made using genetics with zebrafish containing a null mutation in *arylalkylamine-N-acetyltransferase (aanat2*), the rate-limiting enzyme in melatonin biosynthesis [6••]. *aanat2*$^{-/-}$ larvae show dramatically reduced sleep at night, but normal sleep during the day, demonstrating a clear role for endogenous melatonin in promoting nighttime sleep in a diurnal vertebrate. Importantly, behavioral and molecular circadian rhythms are normal in *aanat2*$^{-/-}$ fish, suggesting that melatonin is not required for the establishment or maintenance of normal circadian rhythms, but rather acts downstream of the circadian clock. Endogenous melatonin has been proposed to regulate sleep indirectly by impinging upon the circadian clock, either by phase shifting [7] or by inhibiting the circadian drive for wakefulness [8]. However, when raised under constant dark conditions, in which behavioral and molecular circadian rhythms are absent [9], *aanat2*$^{-/-}$ larvae maintain their decreased sleep phenotype [6••], suggesting that melatonin promotes sleep independently of the clock. Rather, melatonin appears to determine when sleep occurs during the circadian cycle. Indeed, *aanat2*$^{-/-}$ larvae raised in normal light/dark conditions, and transferred to constant dark to remove the masking effects of light on sleep behavior, lose all sleep rhythmicity but maintain locomotor and molecular rhythms [6••]. This striking observation indicates that melatonin is required for circadian regulation of sleep, suggesting that it is the key factor that mediates process C [10], that is, the circadian gating of sleep (Figure 2). It will be interesting to repeat this series of experiments in a diurnal mammal to investigate whether these observations are specific to zebrafish or apply broadly to diurnal vertebrates.

Hypocretin

The neuropeptide hypocretin (Hcrt) is an important regulator of arousal [11]. Hcrt is expressed in thousands of neurons in the mammalian lateral hypothalamus [12,13] that project to arousal centers throughout the brain [14]. In mammals, loss of Hcrt expressing neurons results in narcolepsy [15–17], whereas activation of these neurons promotes sleep to wake transitions [18], demonstrating the crucial role of Hcrt in sleep regulation. Hcrt has been of particular importance for zebrafish sleep research. It was initially the subject of proof-of-principle studies that sought to establish zebrafish as a useful model system for the genetic dissection of sleep. More recently, the zebrafish has returned the favor by providing novel insights into how Hcrt neurons are generated, and how Hcrt promotes wakefulness.

Hcrt research in zebrafish began with genomic and anatomical studies demonstrating conservation between zebrafish and mammals. Zebrafish have a single *hcrt* homolog [19] that is expressed in a small hypothalamic population (~ 10 cells) starting around 24 hours of development [20,21]. In larvae, Hcrt expressing cells send widespread ascending and descending projections throughout the brain, including projections to areas associated with arousal, such as the noradrenergic locus coeruleus (LC) and dopaminergic diencephalic cells, areas which also express the *hypocretin receptor* (*hcrtr*) [21••]. An early study showed sleep fragmentation for $hcrtr^{-/-}$ adult zebrafish, similar to narcoleptic mammals, but also claimed that Hcrt has a sleep-promoting role in zebrafish [22•]. Since then, extensive work has established a wake-promoting role for Hcrt in zebrafish, similar to mammals. First, overexpression of *hcrt* in larvae using a heat-shock inducible promoter results in increased locomotor activity, consolidation of active states, and reduced sleep [21••]. Second, a recently developed high-throughput and non-invasive optogenetic assay demonstrated that stimulation of Hcrt neurons increases locomotor activity (Figure 1C) in a *hcrtr* dependent manner [23••], consistent with the arousing effect of optogenetic stimulation of Hcrt neurons in mice [18]. Third, chemogenetic stimulation of Hcrt neurons, again in freely-behaving zebrafish larvae, using the capsaicin-activated TRPV1 channel increases activity and reduces sleep [24•]. Fourth, real-time monitoring of the activity levels of Hcrt neurons in freely behaving zebrafish larvae revealed that Hcrt neurons are wake active, similar to previous findings in rats and mice [25–27]. Finally, ablation of Hcrt neurons in zebrafish larvae results in increased and fragmented sleep [24•,28], reminiscent of narcolepsy endophenotypes.

Zebrafish research has recently provided insights into how Hcrt neurons are generated and how they promote arousal. The transcription factor Lhx9 was shown to be required for the specification of Hcrt neurons in mice, with $lhx9^{-/-}$ animals having reduced numbers of Hcrt neurons and sleeping more than sibling controls [29]. Starting from a list of transcripts enriched in zebrafish Hcrt neurons, Lhx9 was independently shown to be necessary for Hcrt neuron specification in zebrafish larvae [30•]. In addition, overexpression of *lhx9* in zebrafish larvae resulted in the generation of ectopic Hcrt neurons that express appropriate molecular markers and project to the LC, similar to entopic Hcrt neurons. *lhx9* overexpression in embryonic mice, using *in utero* electroporation, also resulted in specification of additional Hcrt neurons, suggesting that the function of Lhx9 in specifying Hcrt neurons is conserved between fish and mammals [30•]. Interestingly, the *hcrt* promoter contains Lhx9 binding sites that are essential for *hcrt* expression and can be bound by Lhx9 *in vitro* [30•]. These results suggest that Lhx9 both specifies Hcrt neuron identity and directly promotes *hcrt* expression.

Hcrt neurons project to the LC [14], and arousal induced by optogenetic stimulation of Hcrt neurons is suppressed by optogenetic silencing of LC neurons in rodents [31], suggesting that norepinephrine (NE), the main neurotransmitter of the LC, could be a critical mediator of Hcrt-induced arousal. Despite abundant pharmacological evidence for a role for NE in arousal [32], the role of endogenous NE had been unclear, as mice mutant for *dopamine beta-hydroxylase* (*dbh*), the rate limiting enzyme in NE biosynthesis, display developmental defects and have produced conflicting reports on the role of NE in sleep [33,34]. In contrast, $dbh^{-/-}$ zebrafish larvae develop normally, are viable and fertile, and show a dramatic

increase in sleep [23••], thus establishing a role for endogenous NE in sleep regulation. Importantly, insomnia induced by Hcrt overexpression or optogenetic stimulation of Hcrt neurons was suppressed in *dbh*$^{-/-}$ larvae, demonstrating that endogenous NE is required for the arousing effects of Hcrt [23••].

The zebrafish pineal gland expresses *hcrtr* and is also innervated by Hcrt neurons [35], similar to mammals [36–38]. Interestingly, application of Hcrt on cultured pineal glands increases melatonin production, while *hcrtr*$^{-/-}$ adult zebrafish show reduced expression of *aanat2*. Combined with the observation that Hcrt is present during the night [39], these results suggest a role for Hcrt in consolidating nighttime sleep by inducing melatonin production during the night, and could explain the sleep fragmentation phenotype of *hcrtr*$^{-/-}$ zebrafish [22•].

Pharmacology

Larval zebrafish are particularly well suited for small molecule screens and pharmacological studies in general: they are small enough to be screened in 96-well plates in an automated fashion, and compounds are simply added to the water and taken up through the skin and gills [40]. The blood-brain barrier is not fully formed at larval stages [41], thus bypassing a major hurdle in CNS accessibility, and the larvae can survive several days using nutrients supplied by the yolk, with no need for feeding. Indeed, much of the early work on zebrafish sleep was based on pharmacological approaches using known sleep regulators such as melatonin [2••], histamine [42,43] and GABA [43].

In the first, and still most comprehensive, screen for sleep regulating compounds in a vertebrate animal, the effects of over 5,000 small molecules on locomotion and sleep architecture were evaluated in zebrafish larvae [44••]. This study demonstrated conserved roles for multiple signaling molecules in sleep regulation (glutamate, GABA, norepinephrine, serotonin, dopamine, histamine, adenosine, melatonin), setting the stage for the use of zebrafish as a model system for investigating the role of these and other neurotransmitters and neuropeptides in sleep regulation. By assigning a behavioral fingerprint to each compound, and using clustering algorithms to group compounds that induce similar behavioral profiles, potential targets were assigned to previously uncharacterized molecules, and novel pathways with potential sleep-regulating roles were uncovered [44••]. Further studies have used zebrafish to investigate how clinically used hypnotics and psychostimulants impinge upon sleep architecture [45,46].

Progress in understanding sleep regulation has the potential to provide insights into several neurological diseases, including autism spectrum disorders (ASDs), which are associated with hyperactivity and sleep disruption [47]. Zebrafish mutant for the two paralogs of *contactin associated protein-like 2* (*CNTNAP2*), a gene strongly associated with ASD [48], display hyperactivity during the night [49••], as does the mouse knockout [48]. The availability of a database containing the behavioral fingerprints of thousands of drugs in zebrafish larvae [44••] enabled the identification of compounds that generate phenotypes correlating and anti-correlating with those of the *cntnap2ab* mutants [49••]. Drugs with known estrogenic activity were found among the top anti-correlating compounds, suggesting

that such compounds could potentially rescue the *cntnap2ab* mutant phenotype. Indeed, biochanin A and β-estradiol were shown to specifically, and acutely, rescue the hyper-activity phenotype [49••]. This study suggests a potential molecular mechanism for the "female protective model" [50] and raises hopes for the eventual development of ASD treatments based on estrogen signaling.

New approaches and genetic pathways

An implicit promise of a novel model system is the potential to provide insights by opening up new avenues of addressing old questions, as well as to uncover novel genetic pathways. The zebrafish has lived up to expectations on both fronts.

Mice mutant for *dopamine beta-hydroxylase* (*dbh*) display embryonic lethality and require supplementation with a NE precursor during development in order to survive [51]. As adults, these animals have produced conflicting reports on the role of NE in sleep [33,34]. In contrast, $dbh^{-/-}$ zebrafish larvae develop normally without the need for exogenous NE supplementation, and show a dramatic increase in sleep [23••] (Figure 1A). Interestingly, $dbh^{-/-}$ zebrafish larvae also show a severely reduced arousal threshold (Figure 1B), a phenotype that was replicated by pharmacologically blocking beta-adrenergic receptor signaling, but not through pharmacological manipulation of alpha1- or alpha2-adrenergic signaling. Although it is surprising that reduction of an arousal-promoting neurotransmitter results in reduced arousal threshold, NE reuptake inhibitors such as atomoxetine are commonly used as a treatment for Attention Deficit/Hyperactivity Disorder [52], presumably reducing hyper-excitability by increasing NE levels. In another case of a developmental phenotype potentially masking sleep phenotypes, mice mutant for one of the two receptors for the hypothalamic neuropeptide QRFP showed severe bone formation defects [53] (the effects of mutating the other receptor or *qrfp* have not been reported). Zebrafish mutant for both QRPF receptors (*grp103a* and *grp103b*) show no obvious developmental defects; instead $grp103a^{-/-}$; $grp103b^{-/-}$ double mutant larvae are more active and sleep less than sibling controls, suggesting a sleep promoting role for QRFP [54•]. Indeed, overexpression of QRFP reduces activity in a *grp103a; grp103b* dependent manner [54•]. It is worth noting that previous studies in nocturnal rodents based on intracerebroventricular (ICV) injection of peptides derived from QRFP gave conflicting reports, with some suggesting a locomotion-promoting role [55,56] and some finding no significant effect on locomotion [57,58]. However, these studies were based on invasive ICV injection of *in vitro* synthesized peptides, as opposed to genetic loss-of-function studies, a significant caveat when interpreting these results.

A common theme in the above zebrafish studies is the lack of deleterious phenotypes in zebrafish mutants compared to mice with mutations in the homologous genes. The common ancestor of teleosts underwent an additional round of genome duplication compared to other vertebrates, known as the teleost-specific genome duplication (TSD) [59]. It is thus possible that the narrower range of phenotypes observed in $dbh^{-/-}$ and $grp103a^{-/-}$; $grp103b^{-/-}$ mutants is due to the presence of functionally redundant TSD-generated paralogs. However, despite a fully sequenced genome [60], such paralogs have not been detected, and in the case of $dbh^{-/-}$ larvae, the lack of NE was verified by ELISA [23••]. It has been hypothesized that

dbh$^{-/-}$ mice die *in utero* due to hypoxia caused by defects in heart development [61]. Supplementation of parental diet with dihydroxyphenylserine (DOPS), which can be converted to NE independently of DBH, during embryonic development rescues *dbh*$^{-/-}$ pups, and is not required postnatally. Zebrafish larvae develop externally, supported by nutrients received from the yolk sac, and receive adequate oxygenation by diffusion in the absence of normal heart function [62], which may allow zebrafish mutants to bypass this critical developmental window. Finally, developing zebrafish do not require maternal care or other complex social interactions between parents and offspring that are essential for mammalian development, thus avoiding a cause of perinatal lethality and poor development in some mouse mutants. For example, it has been shown that even *dbh*$^{+/-}$ pups born to *dbh*$^{-/-}$ mothers have increased mortality due to deficits in maternal care [51].

Different approaches have been used to identify new genes involved in sleep regulation using zebrafish. In a recent study, Hcrt neurons were purified from zebrafish *Tg(hcrt:EGFP)* larvae by flow cytometry, and their transcriptional profile was analyzed by RNA-seq [63•]. Candidate genes that showed high expression in Hcrt neurons were anatomically verified using *in situ* hybridization or immunofluorescence, techniques that are particularly well suited to the small and optically transparent zebrafish larvae. Among the verified genes was the voltage-gated potassium channel *kcnh4a*, which was shown to be expressed in all Hcrt neurons [63•]. *kcnh4a*$^{-/-}$ mutants demonstrated mild hyperactivity during the day, and a more robust reduction in nighttime sleep, thus establishing Kcnh4a as a novel sleep-regulating channel [63•].

Forward genetic screens in which the progeny of mutagenized parents are screened for phenotypes of interest have been instrumental in the establishment of zebrafish as a model system for the study of development and simple behaviors [64,65]. More recently, a forward genetic screen provided interesting insights into habituation learning [66]. However, such an approach for the study of sleep presents several challenges. Sleep is a long-term state and thus requires assays that last for several days. Also, similar to humans, even closely related zebrafish larvae exhibit considerable variability in sleep/wake behaviors, with phenotypes apparent only at the population level. Furthermore, subtle developmental defects can give rise to locomotion phenotypes that can easily be misinterpreted as sleep phenotypes. To avoid these issues, a candidate approach using an inducible system was recently employed [67••]. Genes predicted to encode human secreted peptides were cloned downstream of heat-shock inducible elements and injected into zebrafish embryos, giving rise to transient transgenic larvae, which were subsequently assayed for locomotion and sleep phenotypes. By comparing behavior before and after heat-shock in the same animals, the authors were able to rapidly screen over a thousand genes that encompass one-third of the human secretome without affecting development, and without the need to produce stable transgenic lines. Over-expression of the neuropeptide neuromedin U (Nmu) was shown to induce a dramatic increase in locomotion, and a concomitant decrease in sleep, with both phenotypes depending upon *nmu receptor 2*; conversely, *nmu*$^{-/-}$ larvae and adults displayed reduced activity [67••]. Interestingly, the Nmu overexpression phenotype correlated with activation of brainstem corticotropin releasing hormone (*crh*) neurons and was blocked by a CRH receptor 1 antagonist, suggesting a novel hypothalamus to hindbrain neuronal and genetic circuit that promotes arousal [67••].

More targeted candidate approaches can also be used. In a recent study, the effects of the overexpression of seven neuropeptides on different arousal modalities was assayed, again using a heat-shock inducible system [68]. By subjecting larvae to a variety of stimuli (mechano-acoustic, visual, thermal), the authors were able to dissect the contributions of different arousal systems on different aspects of sensory responsiveness. For example, compared to sibling controls, Hcrt-overexpressing larvae showed a striking increase in their response to a dark-flash stimulus, but no significant changes in their response to mechano-acoustic or heat stimuli [68]. These results suggest the existence of multiple arousal-regulating systems dedicated to different sensory inputs. Identifying the neuronal and molecular nature of such regulators, and their specializations, could help to deconstruct a general state of reduced arousal, such as sleep, down to its building blocks.

What lies ahead

As sleep research moves from genes to neuronal circuits, zebrafish larvae offer a powerful system for such inquiries. Advances in light-sheet microscopy allow whole-brain functional imaging of zebrafish larvae at cellular resolution using genetic reporters of neuronal activity such as GCaMP6 [69••]. By combining this imaging approach with the pharmacologic, genetic, optogenetic and chemogenetic tools featured in the studies outlined here, zebrafish provide an opportunity to identify novel sleep-regulating circuits and investigate how they interact with known sleep centers. The ability to visualize and manipulate these neurons in real time will allow us to understand the choreography of sleep: how animals enter sleep, how the sleep state is maintained and how it is reversed. Once we truly understand how, we can start offering educated guesses as to why, thus moving closer to unraveling the fascinating mystery that is sleep.

Acknowledgments

We thank Catherine Oikonomou, Daniel Lee and Chanpreet Singh for comments on the manuscript. This work was supported by grants from the National Institutes of Health (NS070911, NS094390 and NS095824), the Mallinckrodt Foundation, the Rita Allen Foundation, and the Brain and Behavior Research Foundation to D.A.P., and from the National Institute of Health (NS082010) and the Della Martin Foundation to G.O.

References

1. Kazimi N, Cahill GM. Development of a circadian melatonin rhythm in embryonic zebrafish. Brain Res Dev Brain Res. 1999; 117:47–52. [PubMed: 10536231]

2••. Zhdanova IV, Wang SY, Leclair OU, Danilova NP. Melatonin promotes sleep-like state in zebrafish. Brain Res. 2001; 903:263–268. The first demonstration that zebrafish larvae exhibit the behavioral criteria for sleep. [PubMed: 11382414]

3. Zhdanova IV. Melatonin as a hypnotic: pro. Sleep Med Rev. 2005; 9:51–65. [PubMed: 15649738]

4. Goto M, Oshima I, Tomita T, Ebihara S. Melatonin content of the pineal gland in different mouse strains. J Pineal Res. 1989; 7:195–204. [PubMed: 2769571]

5. Brzezinski A, Vangel MG, Wurtman RJ, Norrie G, Zhdanova I, Ben-Shushan A, Ford I. Effects of exogenous melatonin on sleep: a meta-analysis. Sleep Med Rev. 2005; 9:41–50. [PubMed: 15649737]

6••. Gandhi AV, Mosser EA, Oikonomou G, Prober DA. Melatonin Is Required for the Circadian Regulation of Sleep. Neuron. 2015; This work identifies melatonin as the mediator of Process C in the two-process model for sleep regulation in zebrafish (see ref 34). doi: 10.1016/j.neuron.2015.02.016

7. Arendt J. Importance and relevance of melatonin to human biological rhythms. J Neuroendocrinol. 2003; 15:427–431. [PubMed: 12622845]

8. Scheer FAJL, Czeisler CA. Melatonin, sleep, and circadian rhythms. Sleep Med Rev. 2005; 9:5–9. [PubMed: 15649734]

9. Kaneko M, Cahill GM. Light-dependent development of circadian gene expression in transgenic zebrafish. PLoS Biol. 2005; 3:e34. [PubMed: 15685291]

10. Borbély AA. A two process model of sleep regulation. Hum Neurobiol. 1982; 1:195–204. [PubMed: 7185792]

11. Sutcliffe JG, de Lecea L. The hypocretins: setting the arousal threshold. Nat Rev Neurosci. 2002; 3:339–349. [PubMed: 11988773]

12. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci US A. 1998; 95:322–327.

13. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell. 1998; 92:573–585. [PubMed: 9491897]

14. Peyron C, Tighe DK, van Den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci. 1998; 18:9996–10015. [PubMed: 9822755]

15. Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM. Reduced number of hypocretin neurons in human narcolepsy. Neuron. 2000; 27:469–474. [PubMed: 11055430]

16. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med. 2000; 6:991–997. [PubMed: 10973318]

17. Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron. 2001; 30:345–354. [PubMed: 11394998]

18. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. Nature. 2007; 450:420–424. [PubMed: 17943086]

19. Kaslin J, Nystedt JM, Ostergård M, Peitsaro N, Panula P. The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. J Neurosci. 2004; 24:2678–2689. [PubMed: 15028760]

20. Faraco JH, Appelbaum L, Marin W, Gaus SE, Mourrain P, Mignot E. Regulation of hypocretin (orexin) expression in embryonic zebrafish. J Biol Chem. 2006; 281:29753–29761. [PubMed: 16867991]

21••. Prober DA, Rihel J, Onah AA, Sung R-J, Schier AF. Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. J Neurosci. 2006; 26:13400–13410. The first demonstration of a role for Hcrt in zebrafish larvae sleep regulation. [PubMed: 17182791]

22•. Yokogawa T, Marin W, Faraco J, Pézeron G, Appelbaum L, Zhang J, Rosa F, Mourrain P, Mignot E. Characterization of sleep in zebrafish and insomnia in hypocretin receptor mutants. PLoS Biol. 2007; 5:e277. The first demonstration of sleep in adult zebrafish and sleep fragmentation in hcrtr $^{-/-}$ adult zebrafish. [PubMed: 17941721]

23••. Singh C, Oikonomou G, Prober DA. Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. Elife. 2015:4. This paper demonstrates the first use of a non-invasive, high-throughput assay for the optogenetic manipulation of genetically-specified neurons in freely behaving animals.

24•. Chen S, Chiu CN, McArthur KL, Fetcho JR, Prober DA. TRP channel mediated neuronal activation and ablation in freely behaving zebrafish. Nat Methods. 2016; 13:147–150. This study describes a novel chemogenetic technique for the activation or ablation of genetically targeted neurons in freely behaving zebrafish larvae. [PubMed: 26657556]

25. Lee MG, Hassani OK, Jones BE. Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. J Neurosci. 2005; 25:6716–6720. [PubMed: 16014733]

26. Mileykovskiy BY, Kiyashchenko LI, Siegel JM. Behavioral correlates of activity in identified hypocretin/orexin neurons. Neuron. 2005; 46:787–798. [PubMed: 15924864]

27. Takahashi K, Lin J-S, Sakai K. Neuronal activity of orexin and non-orexin waking-active neurons during wake-sleep states in the mouse. Neuroscience. 2008; 153:860–870. [PubMed: 18424001]

28. Elbaz I, Foulkes NS, Gothilf Y, Appelbaum L. Circadian clocks, rhythmic synaptic plasticity and the sleep-wake cycle in zebrafish. Front Neural Circuits. 2013; 7:9. [PubMed: 23378829]

29. Dalal J, Roh JH, Maloney SE, Akuffo A, Shah S, Yuan H, Wamsley B, Jones WB, de Guzman Strong C, Gray PA, et al. Translational profiling of hypocretin neurons identifies candidate molecules for sleep regulation. Genes Dev. 2013; 27:565–578. [PubMed: 23431030]

30•. Liu J, Merkle FT, Gandhi AV, Gagnon JA, Woods IG, Chiu CN, Shimogori T, Schier AF, Prober DA. Evolutionarily conserved regulation of hypocretin neuron specification by Lhx9. Development. 2015; 142:1113–1124. This study identifies Lhx9 as both necessary and sufficient to specify Hcrt neurons in zebrafish, and as sufficient to specify Hcrt neurons in mice. [PubMed: 25725064]

31. Carter ME, Brill J, Bonnavion P, Huguenard JR, Huerta R, de Lecea L. Mechanism for Hypocretin-mediated sleep-to-wake transitions. Proc Natl Acad Sci US A. 2012; 109:E2635–44.

32. Berridge CW, Schmeichel BE, España RA. Noradrenergic modulation of wakefulness/arousal. Sleep Med Rev. 2012; 16:187–197. [PubMed: 22296742]

33. Hunsley MS, Palmiter RD. Norepinephrine-deficient mice exhibit normal sleep-wake states but have shorter sleep latency after mild stress and low doses of amphetamine. Sleep. 2003; 26:521–526. [PubMed: 12938804]

34. Ouyang M, Hellman K, Abel T, Thomas SA. Adrenergic signaling plays a critical role in the maintenance of waking and in the regulation of REM sleep. J Neurophysiol. 2004; 92:2071–2082. [PubMed: 15190089]

35. Appelbaum L, Wang GX, Maro GS, Mori R, Tovin A, Marin W, Yokogawa T, Kawakami K, Smith SJ, Gothilf Y, et al. Sleep-wake regulation and hypocretin-melatonin interaction in zebrafish. Proc Natl Acad Sci US A. 2009; 106:21942–21947.

36. Mikkelsen JD, Hauser F, deLecea L, Sutcliffe JG, Kilduff TS, Calgari C, Pévet P, Simonneaux V. Hypocretin (orexin) in the rat pineal gland: a central transmitter with effects on noradrenaline-induced release of melatonin. Eur J Neurosci. 2001; 14:419–425. [PubMed: 11553292]

37. Fabris C, Cozzi B, Hay-Schmidt A, Naver B, Møller M. Demonstration of an orexinergic central innervation of the pineal gland of the pig. J Comp Neurol. 2004; 471:113–127. [PubMed: 14986306]

38. Zhang S, Blache D, Vercoe PE, Adam CL, Blackberry MA, Findlay PA, Eidne KA, Martin GB. Expression of orexin receptors in the brain and peripheral tissues of the male sheep. Regul Pept. 2005; 124:81–87. [PubMed: 15544844]

39. Zeitzer JM, Buckmaster CL, Parker KJ, Hauck CM, Lyons DM, Mignot E. Circadian and homeostatic regulation of hypocretin in a primate model: implications for the consolidation of wakefulness. J Neurosci. 2003; 23:3555–3560. [PubMed: 12716965]

40. Rennekamp AJ, Peterson RT. 15 years of zebrafish chemical screening. Curr Opin Chem Biol. 2015; 24:58–70. [PubMed: 25461724]

41. Jeong J-Y, Kwon H-B, Ahn J-C, Kang D, Kwon S-H, Park JA, Kim K-W. Functional and developmental analysis of the blood-brain barrier in zebrafish. Brain Res Bull. 2008; 75:619–628. [PubMed: 18355638]

42. Peitsaro N, Sundvik M, Anichtchik OV, Kaslin J, Panula P. Identification of zebrafish histamine H1, H2 and H3 receptors and effects of histaminergic ligands on behavior. Biochem Pharmacol. 2007; 73:1205–1214. [PubMed: 17266939]

43. Renier C, Faraco JH, Bourgin P, Motley T, Bonaventure P, Rosa F, Mignot E. Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. Pharmacogenet Genomics. 2007; 17:237–253. [PubMed: 17496723]

44••. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, Haggarty SJ, Kokel D, Rubin LL, Peterson RT, et al. Zebrafish behavioral profiling links drugs to biological targets and rest/

wake regulation. Science. 2010; 327:348–351. The first and most extensive screen for pharmacological regulators of locomotion and sleep in zebrafish larvae. It demonstrates the extensive conservation in the neuropharmacology of sleep between fish and mammals. [PubMed: 20075256]

45. Sigurgeirsson B, Thorsteinsson H, Arnardóttir H, Jóhannesdóttir IT, Karlsson KA. Effects of modafinil on sleep-wake cycles in larval zebrafish. Zebrafish. 2011; 8:133–140. [PubMed: 21882999]
46. Nishimura Y, Okabe S, Sasagawa S, Murakami S, Ashikawa Y, Yuge M, Kawaguchi K, Kawase R, Tanaka T. Pharmacological profiling of zebrafish behavior using chemical and genetic classification of sleep-wake modifiers. Front Pharmacol. 2015; 6:257. [PubMed: 26578964]
47. Hollway JA, Aman MG. Sleep correlates of pervasive developmental disorders: a review of the literature. Res Dev Disabil. 2011; 32:1399–1421. [PubMed: 21570809]
48. Peñagarikano O, Geschwind DH. What does CNTNAP2 reveal about autism spectrum disorder? Trends Mol Med. 2012; 18:156–163. [PubMed: 22365836]
49••. Hoffman EJ, Turner KJ, Fernandez JM, Cifuentes D, Ghosh M, Ijaz S, Jain RA, Kubo F, Bill BR, Baier H, et al. Estrogens Suppress a Behavioral Phenotype in Zebrafish Mutants of the Autism Risk Gene, CNTNAP2. Neuron. 2016; The authors use a hyperactivity phenotype to identify novel potential treatments for Autism Spectrum Disorders. doi: 10.1016/j.neuron.2015.12.039
50. Baron-Cohen S, Lombardo MV, Auyeung B, Ashwin E, Chakrabarti B, Knickmeyer R. Why are autism spectrum conditions more prevalent in males? PLoS Biol. 2011; 9:e1001081. [PubMed: 21695109]
51. Thomas SA, Palmiter RD. Impaired maternal behavior in mice lacking norepinephrine and epinephrine. Cell. 1997; 91:583–592. [PubMed: 9393852]
52. Garnock-Jones KP, Keating GM. Atomoxetine: a review of its use in attention-deficit hyperactivity disorder in children and adolescents. Paediatr Drugs. 2009; 11:203–226. [PubMed: 19445548]
53. Baribault H, Danao J, Gupte J, Yang L, Sun B, Richards W, Tian H. The G-protein-coupled receptor GPR103 regulates bone formation. Mol Cell Biol. 2006; 26:709–717. [PubMed: 16382160]
54•. Chen A, Chiu CN, Mosser EA, Kahn S, Spence R, Prober DA. QRFP and Its Receptors Regulate Locomotor Activity and Sleep in Zebrafish. J Neurosci. 2016; 36:1823–1840. This study identifies QRFP/Gpr103 signaling as necessary and sufficient for sleep in zebrafish larvae. [PubMed: 26865608]
55. Takayasu S, Sakurai T, Iwasaki S, Teranishi H, Yamanaka A, Williams SC, Iguchi H, Kawasawa YI, Ikeda Y, Sakakibara I, et al. A neuropeptide ligand of the G protein-coupled receptor GPR103 regulates feeding, behavioral arousal, and blood pressure in mice. Proc Natl Acad Sci US A. 2006; 103:7438–7443.
56. Do-Régo J-C, Leprince J, Chartrel N, Vaudry H, Costentin J. Behavioral effects of 26RFamide and related peptides. Peptides. 2006; 27:2715–2721. [PubMed: 16730856]
57. Kampe J, Wiedmer P, Pfluger PT, Castaneda TR, Burget L, Mondala H, Kerr J, Liaw C, Oldfield BJ, Tschöp MH, et al. Effect of central administration of QRFP(26) peptide on energy balance and characterization of a second QRFP receptor in rat. Brain Res. 2006; 1119:133–149. [PubMed: 16996040]
58. Moriya R, Sano H, Umeda T, Ito M, Takahashi Y, Matsuda M, Ishihara A, Kanatani A, Iwaasa H. RFamide peptide QRFP43 causes obesity with hyperphagia and reduced thermogenesis in mice. Endocrinology. 2006; 147:2916–2922. [PubMed: 16543370]
59. Meyer A, Schartl M. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr Opin Cell Biol. 1999; 11:699–704. [PubMed: 10600714]
60. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013; 496:498–503. [PubMed: 23594743]
61. Thomas SA, Matsumoto AM, Palmiter RD. Noradrenaline is essential for mouse fetal development. Nature. 1995; 374:643–646. [PubMed: 7715704]

62. Pelster B, Burggren WW. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (Danio rerio). Circ Res. 1996; 79:358–362. [PubMed: 8756015]

63•. Yelin-Bekerman L, Elbaz I, Diber A, Dahary D, Gibbs-Bar L, Alon S, Lerer-Goldshtein T, Appelbaum L. Hypocretin neuron-specific transcriptome profiling identifies the sleep modulator Kcnh4a. Elife. 2015; 4 The authors use RNA-seq of Hcrt neurons to identify a potassium channel whose loss results in reduced sleep in zebrafish larvae.

64. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, et al. A genetic screen for mutations affecting embryogenesis in zebrafish. Development. 1996; 123:37–46. [PubMed: 9007227]

65. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, et al. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development. 1996; 123:1–36. [PubMed: 9007226]

66. Wolman MA, Jain RA, Marsden KC, Bell H, Skinner J, Hayer KE, Hogenesch JB, Granato M. A genome-wide screen identifies PAPP-AA-mediated IGFR signaling as a novel regulator of habituation learning. Neuron. 2015; 85:1200–1211. [PubMed: 25754827]

67••. Chiu CN, Rihel J, Lee DA, Singh C, Mosser EA, Chen S, Sapin V, Pham U, Engle J, Niles BJ, et al. A Zebrafish Genetic Screen Identifies Neuromedin U as a Regulator of Sleep/Wake States. Neuron. 2016; 89:842–856. This study uses transient transgenesis to perform the first large-scale genetic screen for vertebrate sleep regulators. [PubMed: 26889812]

68. Woods IG, Schoppik D, Shi VJ, Zimmerman S, Coleman HA, Greenwood J, Soucy ER, Schier AF. Neuropeptidergic signaling partitions arousal behaviors in zebrafish. J Neurosci. 2014; 34:3142–3160. [PubMed: 24573274]

69••. Ahrens MB, Orger MB, Robson DN, Li JM, Keller PJ. Whole-brain functional imaging at cellular resolution using light-sheet microscopy. Nat Methods. 2013; 10:413–420. This study uses the genetically encoded calcium indicator GCaMP and light sheet microscopy to achieve functional imaging at cellular resolution and 0.8 Hz frequency in zebrafish larvae. [PubMed: 23524393]

70. Cahill GM, Hurd MW, Batchelor MM. Circadian rhythmicity in the locomotor activity of larval zebrafish. Neuroreport. 1998; 9:3445–3449. [PubMed: 9855296]

71. Aho V, Vainikka M, Puttonen HAJ, Ikonen HMK, Salminen T, Panula P, Porkka-Heiskanen T, Wigren H-K. Homeostatic response to sleep/rest deprivation by constant water flow in larval zebrafish in both dark and light conditions. J Sleep Res. 2017; doi: 10.1111/jsr.12508

Highlights

- Zebrafish research has offered novel insights into sleep regulation.
- Melatonin is required for circadian regulation of sleep.
- Lhx9 regulates Hcrt cell fate, and norepinephrine mediates Hcrt-induced arousal.
- QRFP and Kcnh4 are novel sleep regulators identified by zebrafish research.
- High-throughput tracking, optogenetics and whole-brain imaging promise further discoveries.

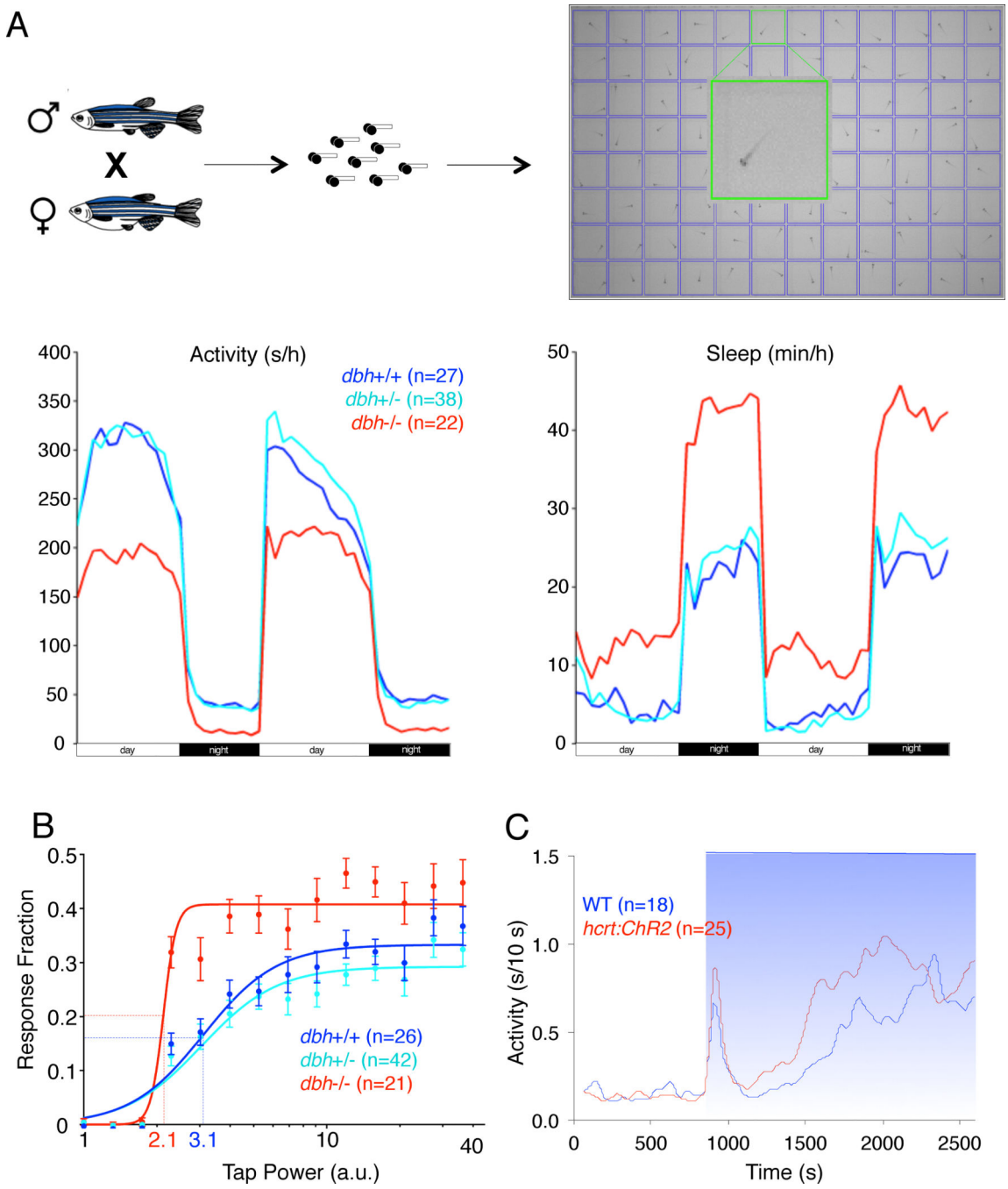

Figure 1. Common zebrafish sleep assays: Monitoring locomotor activity and sleep, determining arousal threshold and quantifying results of optogenetic manipulations

(**A**) Quantification of locomotor activity and sleep. Zebrafish larvae are housed in wells of a 96-well plate and their activity is monitored using an infrared camera, giving rise to locomotor activity and sleep graphs. In this example, animals lacking endogenous norepinephrine due to a mutation in *dopamine beta-hydroxylase* (*dbh*) show reduced activity and increased sleep. (**B**) Determining arousal threshold. The response of larvae to mechano-acoustic stimuli of varying intensities is quantified, and a stimulus-response curve is

constructed in order to determine the tapping power at which 50% of the maximal response is achieved (Effective Tap Power 50, ETP_{50}). *dbh*$^{-/-}$ animals show a reduced arousal threshold (ETP_{50} = 2.1 for *dbh*$^{-/-}$ vs. 3.1 for *dbh*$^{+/+}$ sibling control). a.u. = arbitrary units. (**C**) Optogenetic manipulation of neuronal activity. Upon exposure to ambient blue light during the dark phase (blue-shaded area), all larvae respond with an acute startle, followed by near return to baseline and finally a lasting increase in locomotion as they adapt to the new light conditions. Animals expressing ChR2 in Hcrt neurons show a significantly enhanced response compared to sibling controls throughout the trial.

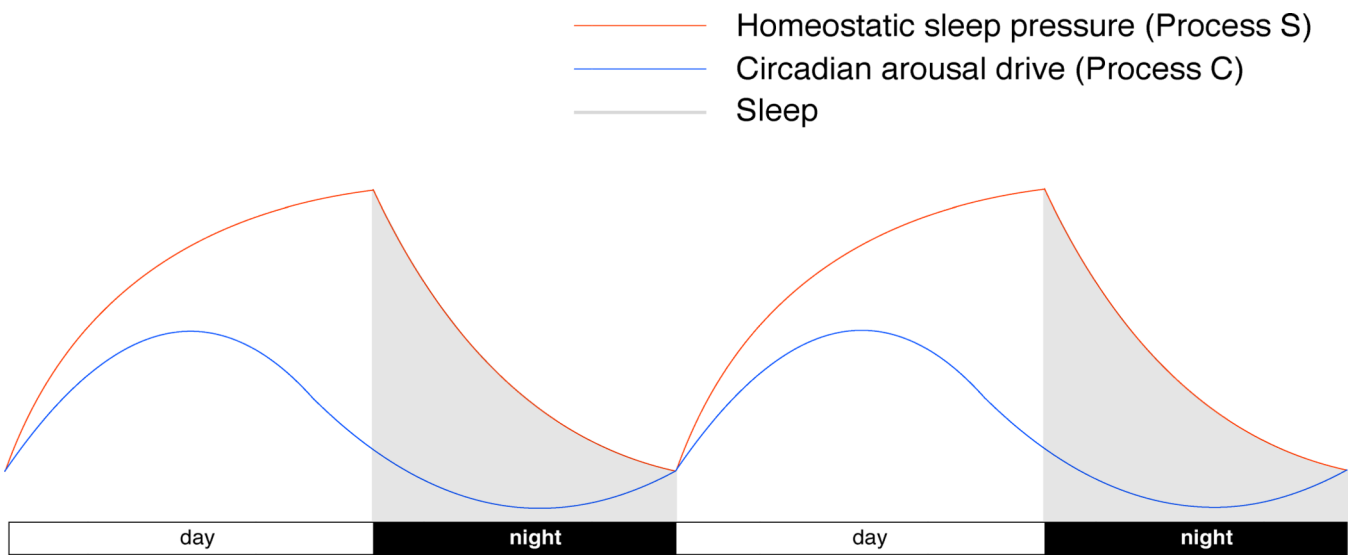

Figure 2. The two-process model of sleep regulation

Sleep is regulated by homeostatic drive (Process S) and circadian gating (Process C). In diurnal animals, during the day homeostatic sleep pressure continuously increases, while the circadian drive for wakefulness is initially increased and then reduced as night approaches. When the difference between the two surpasses a threshold, sleep entry occurs. Homeostatic pressure is relieved during sleep, while the circadian drive for activity begins to rise as morning approaches, facilitating exit from the sleep state. Based on [10].